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14. ABSTRACT

We have performed multiple phage screens in search for carcinoma-associated fibroblast (CAF)-targeting peptides utilizing the tumor-penetrating phage display technology combined with next generation phage DNA sequencing. We have identified at least 4 potentially novel CAF/stroma-targeting peptides. One of the peptides, CIS, homed to breast tumor stroma in two mouse models, and the homing was significantly enhanced by iRGD co-injection.

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2014 REPORT
PROBING TUMOR MICROENVIRONMENT WITH IN VIVO PHAGE DISPLAY

Introduction:
Tumor microenvironment is critical to tumor development and progression. One of the components, tumor vasculature, has caught much attention and has become a major target in cancer therapy (Ruoslahti et al., 2010). Other components include immune cells (e.g., macrophages), mesenchymal cells (e.g., fibroblasts), and extracellular matrix. Macrophages produce growth factors and cytokines, which can promote tumor cell growth and angiogenesis (Pollard, 2004; Chen et al., 2005). Tumor fibroblasts also promote tumor growth, and produce extracellular matrix that blocks the access of anti-tumor drugs to tumor cells (Kalluri & Zeisberg, 2006; Sund & Kalluri, 2009). These macrophages and fibroblasts (and other stromal cells in tumor microenvironment) are thought to express markers not present in their normal counterparts. The goal of this project is to develop new probes for various cell populations in the tumor microenvironment. To accomplish the goal, we will develop a novel phage display technology that extends the reach of phage particles beyond the tumor vasculature to probe extravascular space in tumors. We will co-apply phage library with iRGD, a tumor-penetrating peptide, which facilitates penetration of co-applied molecules into tumor tissue (Sugahara et al, 2009; 2010).

Body:
The *Specific Aims* approved for the study are:

Aim 1. Perform phage library screenings to identify homing peptides for individual cell types in tumors by using new tumor-penetrating screening technology.

Task 1a. Perform tumor-penetrating screens for tumor-homing peptides on orthotopic and MMTV-PyMT *de novo* breast tumors. (Months 1-12)

Responsible PI
Erkki Ruoslahti: screens on cancer stem cells and tumor-associated macrophages
Kazuki Sugahara: screens on carcinoma-associated fibroblasts

Task 1b. Perform tumor-penetrating screens for tumor-homing peptides in human breast cancer explants. (Months 1-12)

Responsible PI
Erkki Ruoslahti: screens on cancer stem cells and tumor-associated macrophages
Kazuki Sugahara: screens on carcinoma-associated fibroblasts
Sarah Blair: collection of human breast tumor explants

Task 1c. Optimize and validate the experimental approach and custom-made bioinformatics software for high throughput phage sequencing. (Months 1-12)

Responsible PI
Kazuki Sugahara

Aim 2. To validate the homing specificities of individual phage and synthetic peptides from Task 1 in ex vivo and in vivo tests.

Task 2a. Analyze the homing specificity of homing peptides recognizing tumor fibroblasts. (Months 13-24)

Responsible PI
Kazuki Sugahara

Task 2b. Analyze the homing specificity of homing peptides recognizing tumor-associated macrophages (Months 13-24).

Responsible PI
Erkki Ruoslahti

Task 2c. Analyze the homing specificity of homing peptides recognizing cancer stem cells (Months 13-24).

Responsible PI
Erkki Ruoslahti

Results

Aim 1.

We have created a breast tumor mouse model by orthotopically injecting MCF10CA1a human breast cancer cells into nude mice. The MCF10CA1a tumors contain high amounts of tumor stroma, which iRGD efficiently penetrates through (refer to Fig. 8). We have also established immortalized human breast CAF lines, hb6008 and hb6011. The two lines have been labeled with mCherry to allow isolation of the cells from tumors made by co-implantation of the CAFs and breast tumor cells (Fig. 1). We have also collected a number of human breast tumor samples with help of our collaborator, Dr. Sarah Blair at the University of California, San Diego. We have collected 5 primary tumors, 2 metastatic lymph nodes, and 2 normal breast tissues. Fluorescein-labeled iRGD (FAM-iRGD) and co-applied phage particles efficiently penetrated into all the human tumor explants tested, but not into normal breast tissue (Fig. 2). FAM-CRGDC, a non-tissue penetrating control peptide, did not facilitate phage penetration into the tumors.

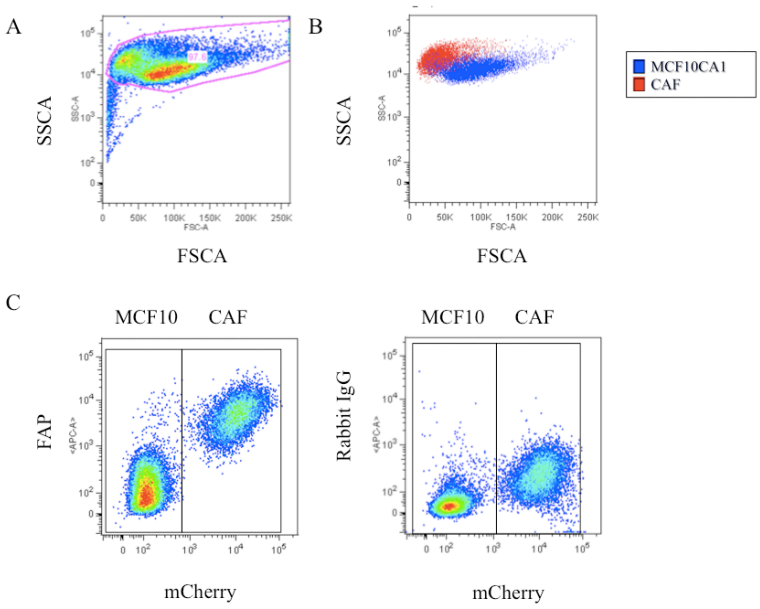

Fig. 1. Separation of CAFs from MCF10CA1a breast tumor cells. MCF10CA1a human breast tumor cells and mCherry-labeled hb6011 CAFs were mixed *in vitro*, and the CAFs were separated based on mCherry expression using flow cytometry. (A) Forward (FSC) and side (SSC) scatter plot of the mixed population. (B) FSC and SSC plot showing the two different populations CAFs in red and MCF10CA1 in blue based on the mCherry gates plotted in (C). (C) Dot plots showing mCherry expression on the X axis and fibroblast activation protein (FAP) or rabbit isotype control staining in the Y axis. Note that CAFs are potentially distinguishable using FSC and SSC alone.

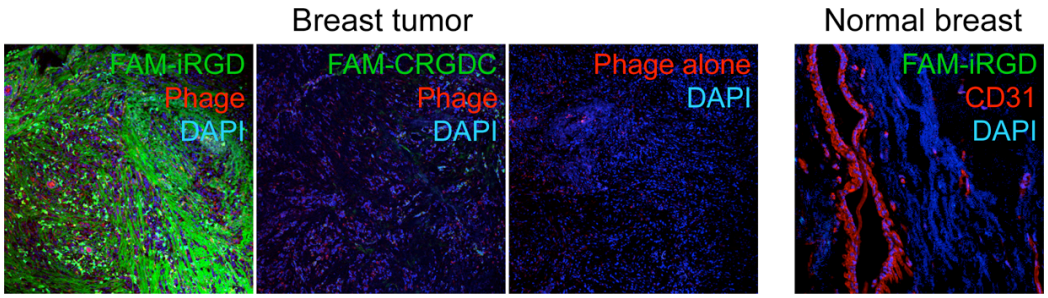

Fig. 2. Co-penetration of iRGD and phage into human breast tumor explants. Fresh surgical samples of human breast tumors or normal breast tumors that were resected together with the tumors were maintained in short-term culture in the presence of FAM-iRGD (green) and phage expressing inert G_7 peptide for 90 min. The tumors were washed, fixed, and processed for confocal microscopy. Red, phage (except for the right most panel where red represents blood vessels); blue, nuclei.

We optimized two critical techniques required for successful phage display: (1) CAF isolation from tumor explants, and (2) high throughput sequencing (HTS) for phage DNA. We have established a method to isolate CAFs from breast tumor explants. Tumors are chopped into small pieces with surgical blades, and treated with collagenase for 45 minutes. The crude cell suspension is washed in culture media, passed through a cell strainer, and CAFs are isolated on a magnetic column or by flow cytometry-based cell sorting using an antibody against fibroblast activation protein (FAP). During the optimization steps, flow cytometry revealed that 30-40% of total cells in a MCF10CA1a tumor are CAFs (refer to Fig. 10).

HTS and bioinformatics function as shown in Fig. 3. Briefly, phage DNA is purified, and subjected to emulsion PCR using primers with Ion Torrent adapters for clonal amplification on Ion Sphere Particles. The particles are isolated, loaded on a chip, and sequenced using an Iron Torrent next generation sequencer. A test run on a naïve phage library yielded highly diverse CX_7C peptide sequences. This method allows us to acquire peptide sequences of the entire phage pool to probe the full landscape of the homing peptides recovered. Thus, only a single round of selection is required for a screen to be informative, and eliminates any bias caused by differences in amplification rates of the phage clones.

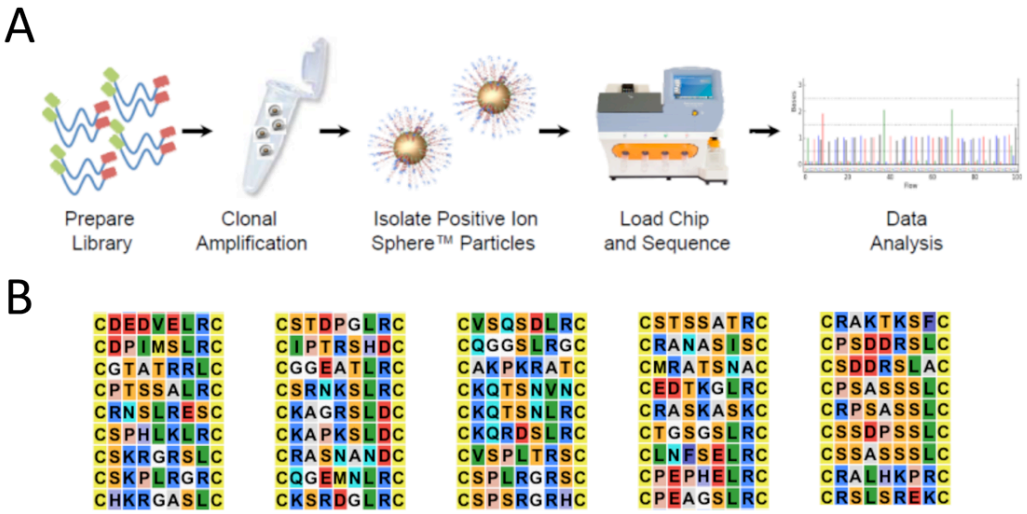

Fig. 3. Phage DNA sequencing with Iron Torrent next generation sequencer. (A) Work flow of the sequencing procedure. After phage panning, phage particles are recovered for DNA purification. A DNA library is prepared using fusion primers with Ion Torrent adapters, and clonally amplified on Ion Sphere Particles by emulsion PCR. The particles are isolated, loaded on a chip, and subjected for sequencing with an Ion Torrent machine. (B) Example of peptide sequences in a naïve CX_7C library detected with Ion Torrent. Amino acids are color-coded based on their chemical characteristics.

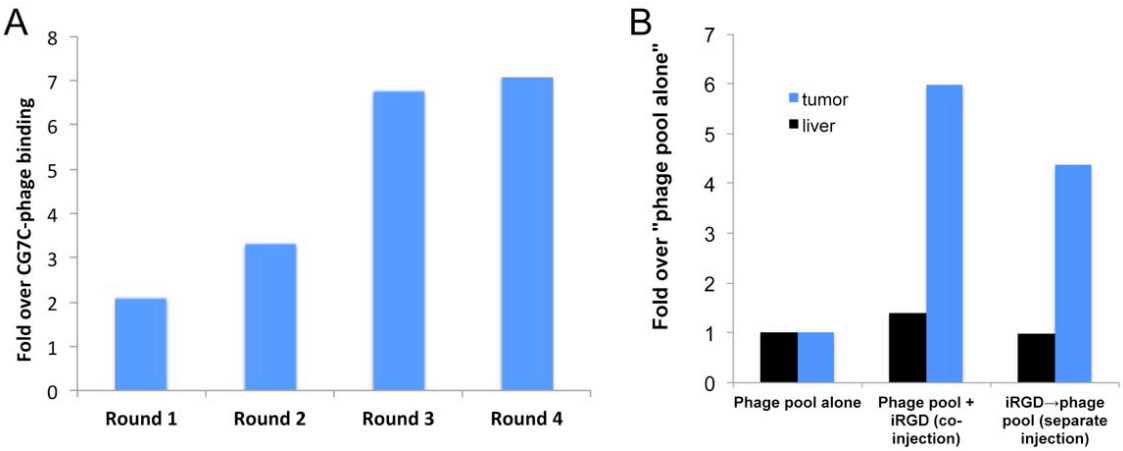

Fig. 4. *In vitro* and *in vivo* phage screen for CAF binding peptides. A, A T7 phage pool expressing a cyclic CX_7C peptide library was applied to cultured i6011 immortalized human breast CAFs to enrich phages that

bind to the CAFs. **B**, The phage pool from "Round 3" in panel A was injected alone, together with 75 μg of iRGD, or 15 min after iRGD injection into the tail vein of mice bearing orthotopic MCF10CA1a human breast cancer xenografts. Phages that accumulated in the tumor (and liver as a control) were recovered and titrated. Note that iRGD facilitated phage accumulation into the tumor but not into the liver.
The phage pool after the 3rd *in vitro* panning was intravenously injected into mice bearing orthotopic MCF10CA1a human breast tumor xenografts. In some cases, the pool was injected together with synthetic iRGD peptide or 15 min after iRGD injection to facilitate extravasation of the phage pool within the breast tumor tissue. Co-injection of iRGD enhanced phage accumulation in the tumor by 4-6 fold, but minimally affected phage entry into the liver (B).

To identify novel breast CAF-targeting peptides, we have performed various types of phage display screens (e.g., *in vitro* + *in vivo*, *ex vivo* with human tumor samples, *in vivo*). Among them, a combination of *in vitro* and *in vivo* display has yielded peptides that potentially target breast CAFs.

First, four rounds of *in vitro* phage panning on cultured breast CAFs were performed. A cyclic CX_7C peptide library displayed on T7 phage was used (diversity approximately 10^9). The CAFs, i6011, were generated by immortalizing hb6011 using human telomerase reverse transcriptase (hTERT). The resulting phage pool bound to i6011 approximately 7 fold more than a phage clone expressing an inert peptide, CG_7C (Fig. 4A).

A

Phage	Phage pool alone	Co-injection	Separate injection
CISQERGESC	511	0	0
CIFSGEGESC	202	0	0
CAVDGGSRC	48	0	0
CKRKDSRSC	31	0	0
CKTKDGRNC	11	0	0
CRLDKKGDC	11	0	0
LGRGGVAKL	11	0	0
CPPEARKRC	11	0	0
CQLGRSQKC	10	0	0
CKRTPDSKC	10	0	0
CTGTAHSKC	10	0	0
CNTGARSRC	10	0	0
CRTNGVKAC	9	0	0
CDLNTDSPC	9	0	0
CMGKGKKPC	9	0	0
CTEKMSQKC	9	0	0
CRSSAKL	9	0	0
[illegible]	8	0	0

B

Phage	Phage pool alone	Co-injection	Separate injection
CQEKTKNRC	0	1782	0
CAVRQKGEC	0	59	2440
CHKR	0	8	5
CAVD	0	8	5
CGAD	0	8	2
CPH	0	7	4
CRGDKGPDC	0	7	3
CNGREVSSC	0	7	0
CERP	0	7	0
CDLA	0	6	3
CKTPR	0	6	2
L	0	6	2
CPKL	0	6	0
CPKP	0	6	0
CSGSPQRKC	0	6	0
CAAS	0	5	4
CREPR	0	5	3
[illegible]	0	5	3

Table 1. Peptides recovered from the tumors analyzed by high throughput phage DNA sequencing technology. A, Peptides found in the "Phage pool alone" group are listed in a descending order of frequency. Note that CISQERGESC (CIS) and CIFSGEGESC (CIF) with relevant amino acid sequences were highly enriched in the pool. The total number of phage clones analyzed was 90347, and 57810 peptides were found. **B**, Peptides found in the "Co-injection (simultaneous injection of iRGD/phage pool)" group are listed in a descending order of frequency. CQEKTKNRC (CQE) and CAVRQKGEC (CAV) were highly enriched in the pool. Notably, CAV was the most enriched peptide in the "Separate injection (iRGD followed by phage pool injection)" group. In addition, CRGDKGPDC, the iRGD peptide sequence, was found in both groups. The total number of phage clones analyzed for the "Co-injection" group was 94674, and 70383 peptides were found. A total of 97638 phage clones were analyzed for the "Separate injection" group, and 73745 peptides were found.

Phage particles recovered from the tumor tissue were then subjected to DNA sequencing using an Iron Torrent next generation sequencer. The phage pool recovered from the MCF10CA1a tumor in the mouse that received phage pool alone contained 511 phage clones expressing CISQERGESC (CIS: 0.6% of the recovered phage clones) and 202 phages expressing a relevant peptide CIFSGEGESC (CIF: 0.2%) (Table 1A). The phage pool from the iRGD/phage simultaneous injection screen contained 1782 phage clones expressing CQEKTKNRC (CQE: 1.8%) (Table 1B). The phage pool from the screen that involved phage injection after iRGD injection contained 2440 phages expressing CAVRQKGEC (CAV: 2.6%). The CAVRQKGEC phage was also present in the iRGD/phage simultaneous injection group (0.1%). Interestingly, phages expressing the iRGD sequence (CRGDKGPDC) were detected in the iRGD co-injection screens supporting our finding that iRGD is a CAF-targeting peptide (see Fig. 11).

Aim 2.

Fluorescein (FAM)-labeled CIS was synthesized in house as described earlier (Sugahara et al, 2009). Synthetic FAM-CIS injected intravenously into mice bearing orthotopic MCF10CA1a tumors specifically accumulated in the tumor tissue (Fig. 5A). FAM-CIS also homed to breast tumors in MMTV-PyMT transgenic mice (Fig. 5B). Co-injection of non-labeled iRGD synthetic peptide enhanced the tumor homing efficiency and specificity of FAM-CIS in MMTV-PyMT mice.

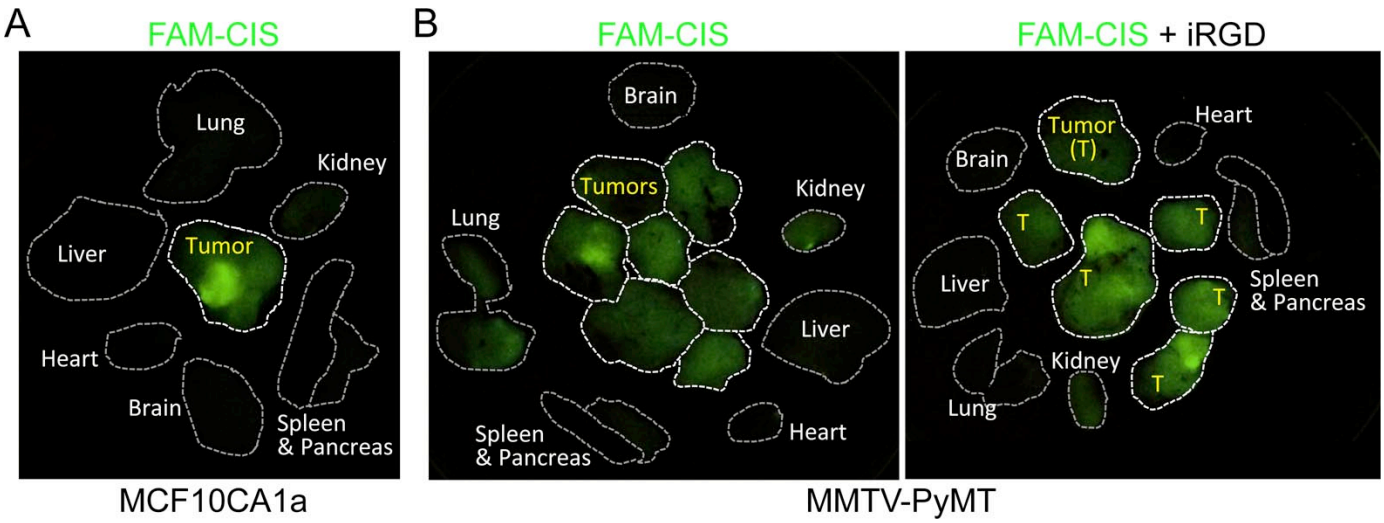

Fig. 5. *In vivo* distribution of fluorescein-labeled synthetic CIS peptide (FAM-CIS). Approximately 150 μg of synthetic FAM-CIS peptide were intravenously injected into mice bearing orthotopic MCF10CA1a human breast cancer xenografts (**A**) and transgenic MMTV-PyMT mice bearing *de novo* breast tumors (**B**). In some cases, non-labeled synthetic iRGD peptide was co-injected with FAM-CIS. After 1 hr of peptide circulation, the mice were perfused through the heart with PBS, and tumors and tissues were collected for macroscopic imaging under a fluorescent light table. The dotted lines outline the tissues. Note the tumor-specific accumulation of FAM-CIS. iRGD appears to enhance the tumor homing efficiency and specificity of FAM-CIS.

Immunofluorescence revealed that FAM-CIS targets tumor blood vessels, and subsequently, the extravascular tumor stroma in both orthotopic MCF10CA1a xenograft and *de novo* MMTV-PyMT tumors (Fig. 6). This finding supports the possibility that CIS is a CAF-binding peptide. In addition to the vasculature and stroma, CIS accumulated into distinct cell populations (Fig. 6, arrows) within the breast tumor tissue.

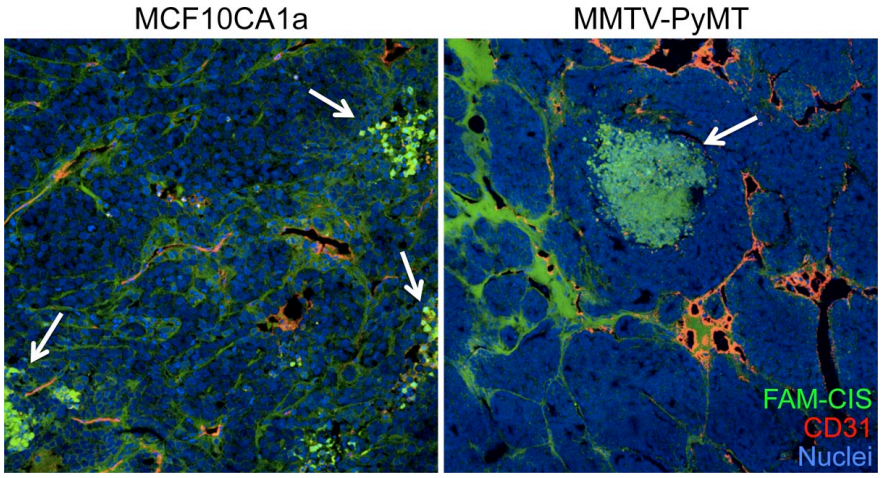

Fig. 6. Intratumoral distribution of Intravenously injected FAM-CIS. Confocal images of tumors from mice intravenously injected with FAM-CIS (green) are shown. Red, CD31; blue, DAPI. Note that the CD31 positive tumor vessels and stromal network are highlighted by FAM-CIS. FAM-CIS also accumulated into distinct cell populations within the tumor tissue (arrows).

Co-injection of non-labeled iRGD peptide enhanced the extravasation of FAM-CIS into the tumor parenchyma, suggesting that a combination of CIS and iRGD can be a powerful tool to simultaneously target breast tumor parenchyma and stroma (Fig. 7).

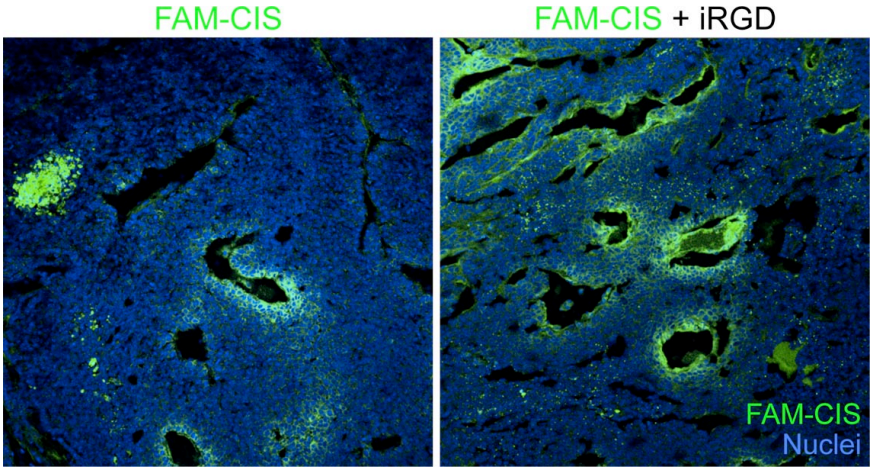

Fig. 7. Effect of iRGD co-injection on the intratumoral distribution of FAM-CIS. Confocal images of *de novo* tumors from MMTV-PyMT transgenic mice that intravenously received FAM-CIS (green) with or without iRGD are shown. Blue, DAPI. Note the enhanced extravasation of FAM-CIS into the tumor parenchyma when iRGD was co-injected.

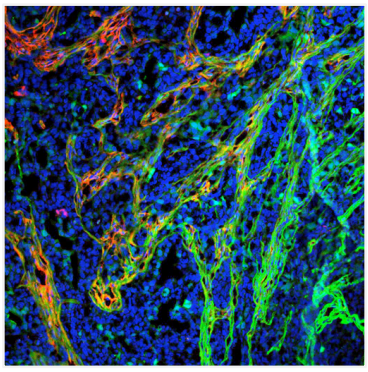

Fig. 8. iRGD targets breast CAFs. Fluorescein (FAM)-labeled iRGD (green) was intravenously injected into mice bearing orthotopic MCF10CA1a human breast tumors, and was allowed to circulate for 30 min. The mice were perfused through the heart with PBS, and the tumors were subjected to immunofluorescence. Red, fibroblast marker (ER-TR7); Blue, nuclei. Note the colocalization of FAM-iRGD and fibroblasts.

While performing the new screens, we have discovered that iRGD itself efficiently targets CAFs in breast tumors. Intravenously injected FAM-iRGD efficiently penetrated breast tumor stroma, and colocalized with CAFs (Fig. 8). iRGD efficiently bound to and internalized into cultured hb6008 and hb6011 CAFs. Flow cytometery revealed that both CAF lines express high levels of iRGD receptors, αv integrins and neuropilin 1 (NRP-1) (Fig. 9).

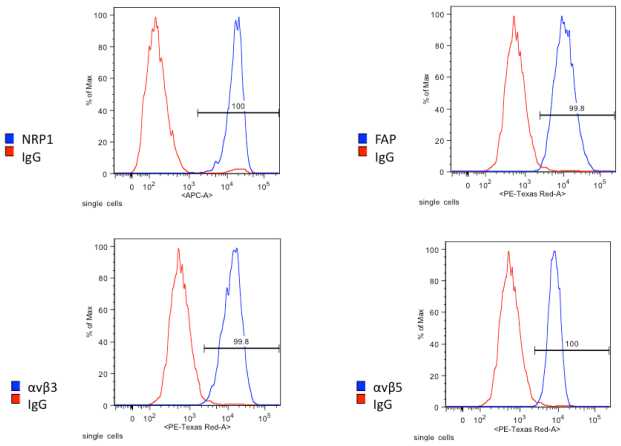

Fig. 9. Expression of iRGD receptors in hb6011 CAFs. The expression of αvβ3 and αvβ5 integrins, neuropilin-1 (NRP-1), and fibroblast activation protein (FAP) in hb6011 CAFs was analyzed by flow cytometry. Isotype control (IgG) is shown in red and the integrins, NRP-1, and FAP are in blue.

High NRP-1 expression was found in CAFs isolated from orthotopic MCF10CA1a tumors. Importantly, NRP-1 expression was particularly high in the CAF population, suggesting its potential as a CAF marker in breast tumors (Fig. 10). In addition, our preliminary results indicate that FAM-iRGD accumulates into a subset of cells in premalignant lesions in the mammary fat pad (Fig. 11). Immunofluorescence revealed that the iRGD signals colocalized with vimentin, a CAF marker (data not shown). These results suggest that iRGD is a CAF-targeting peptide with tumor-penetrating properties and that the iRGD receptor NRP-1 is a potential CAF marker in breast tumors and premalignant breast lesions.

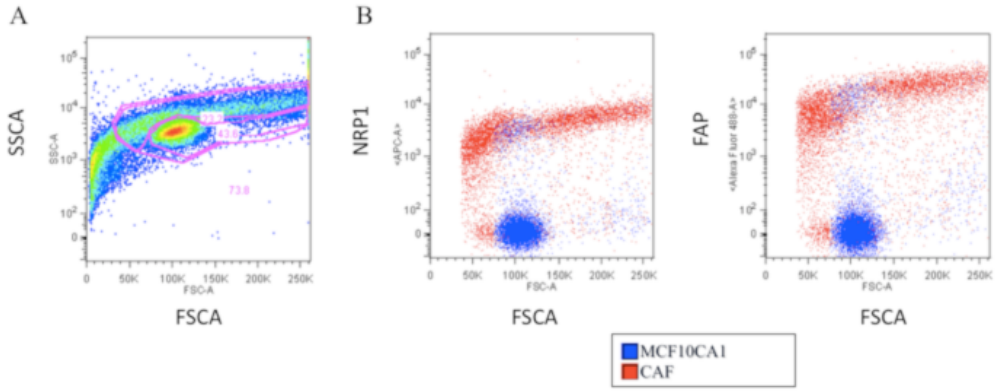

Fig. 10. NRP-1 expression in breast CAFs. (A) FSC and SSC dot plot of tumor cell suspension. The tumor was generated by injection of MCF10CA1 and 6011(2:1) into the mammary fat pad of nude mice. The two gates represent the MCF10CA1 and the CAFs and are based on the data shown on figure 2. (B) Dot plots of NRP-1 and FAP expression in the CAFs (red) and the MCF10CA1 (blue) showing that the CAFs are the highest NRP-1 and FAP expressing population.

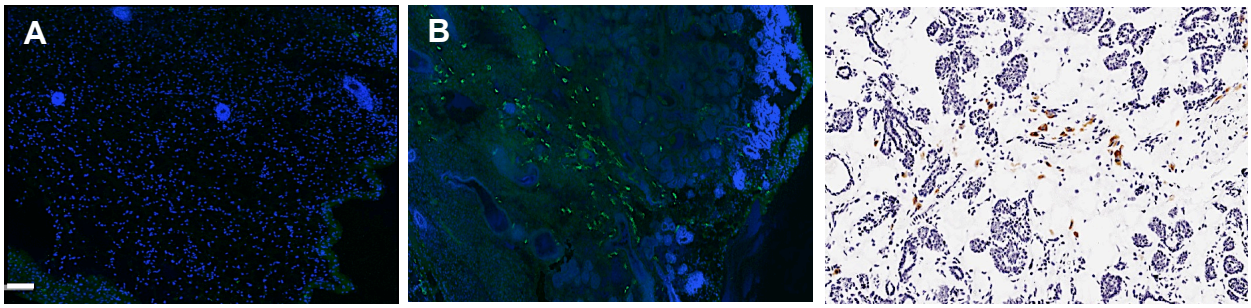

Fig. 11. iRGD homes to early (premalignant) hyperplastic lesions in mammary fat pad isolated from MMTV-PyMT animals. Immunofluorescence on whole mount sections of mammary fat pad isolated

following 1 hour circulation of 0.15 μmol FAM-iRGD in normal Blk6 mouse (**A)** or day 48 MMTV-PyMT mouse **(B**). Green – anti-FAM-iRGD; Blue - Nuclear Stain. Scale Bar -100 μm. **(C)** Anti-FAM staining in mammary fat pad sections isolated from an MMTV-PyMT mouse intravenously injected with FAM-iRGD.

Key Research Accomplishments:

- We have established tools and key technologies required for the proposed phage display project. Those include mouse tumor models, immortalized human breast CAFs, CAF isolation techniques, and high throughput phage DNA sequencing.
- We have demonstrated the utility of the next generation phage DNA sequencing technology.
- Using the tools, we have performed multiple phage screens in search for CAF-targeting peptides.
- We have identified 4 potentially novel CAF-targeting peptides.
- One of the peptides, CIS, was found to home to breast tumors in two different tumor mouse models.
- Intravenously injected CIS peptide efficiently accumulated into breast tumor stroma.
- iRGD co-injection enhances breast tumor targeting of CIS.
- iRGD alone efficiently targets breast CAFs, and the iRGD receptor, NRP-1, is a potential CAF marker in breast tumors.
- iRGD and co-applied phage particles penetrate into human breast tumor explants.

Reportable outcomes:

None.

Conclusions:

We have performed multiple tumor-penetrating phage screens (using iRGD co-injection) in which we have demonstrated the utility of our next generation phage DNA sequencing technology. One of the screens has yielded at least 4 potential CAF-targeting peptides. One of them, CIS appears to target breast tumors, especially tumor stroma, where CAFs mainly reside. Our most recent data acquired after the grant term ended showed that CIS and CIF, which have very similar amino acid sequences, bind to cultured hb6011 CAFs especially to filopodia and fibrous structures extending from the cells (data not shown). Receptor hunt for the peptides is now underway.

In addition to the new screens, we have found that iRGD alone is a CAF-targeting peptide and that the iRGD receptor NRP-1 is a potential CAF marker. Combination of iRGD with CIS can be a powerful tool to target breast tumors. These findings are important not only for CAF-targeting, but also to further understand the origin and functions of CAFs in breast tumors. In addition, our latest data acquired after the grant term ended demonstrate that the presence of CAFs in the tumor microenvironment is critical for the tumor penetrating activities of iRGD (data not shown). We will seek for additional funding to continue the project.

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Appendices:
None